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Fine tuning of insulin secretion by release of nerve growth factor from mouse and human islet β-cells

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Abstract

Nerve growth factor (NGF) is a protein required for neuronal development that also has regulatory functions in non-neuronal cells. Both NGF and its membrane receptors trkA and p75 are expressed by islet β-cells. In this study we dynamically profiled NGF secretion from islets and used selective trkA and p75 inhibitors to identify the role of endogenous NGF in β-cell stimulus-secretion coupling. NGF secretion from mouse islets was transient and did not accompany the sustained second phase of glucose-induced insulin secretion. Despite being present in human islets, NGF was not released at sufficient levels to be quantified. Inhibition of NGF signaling through trkA and $p75^{NTR}$ increased basal insulin secretion from both human and mouse islets, but impaired glucose-stimulated insulin secretion. These data support a role for islet NGF in fine-tuning insulin secretion, to both maintain a low basal level of insulin output and contribute to the biphasic secretory response to glucose.

Highlights

- Glucose stimulates transient NGF secretion from mouse islets
- Disruption of NGF signaling via trk A and $p75^{NTR}$ increases basal insulin secretion
- Inhibition of trkA and $p75^{NTR}$ impairs glucose-induced insulin secretion
- $p75^{NTR}$ binds to β-cell Rho-GDI, which may modulate the amplitude of insulin release

Keywords

NGF, insulin secretion, islets of Langerhans, β-cells, trkA, $p75^{NTR}$

Abbreviations

BMI, body mass index ; ECL, enhanced chemoluminescence; GDI, GDP-dissociation inhibitor; glucose-stimulated insulin secretion, glucose-stimulated insulin secretion; GTP, guanosine tri-phosphate; HRP, horseradish peroxidase; ICD, intracellular domain; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PMA, 4β-phorbol-12-myristate-13-acetate; Rho-GDI, Rho GDP dissociation inhibitor; SDS, sodium dodecyl sulfate; TNF, tumour necrosis factor

1. INTRODUCTION

In addition to insulin, the main regulator of glucose homeostasis, islet β-cells are capable of storing a wide array of peptides and small molecules within their secretory vesicles (Suckale and Solimena, 2010), many of which can act locally at β-cell receptors to exert autocrine and paracrine effects (Amisten et al., 2013). Nerve growth factor (NGF) is one of those peptides that is synthesised and secreted by β-cells in response to appropriate stimuli (Rosenbaum et al., 1998). This peptide was originally identified as a factor necessary for correct neuronal growth and differentiation (Cohen and Montalcini, 1957) and it has since been demonstrated to control a broader range of homeostatic responses both in the central and peripheral nervous systems, as well as in non-neuronal tissues (Kaplan and Miller, 1997).

NGF acts *via* two membrane receptors: the low affinity $p75^{NTR}$, an atypical member of the TNFα superfamily (Zampieri and Chao, 2006), and the high affinity trkA, belonging to the tyrosine kinase receptor family (Muragaki et al., 1995). While trkA works through phosphorylation, mainly regulating the activation of signals promoting cell survival, proliferation and differentiation (Freund and Frossard, 2004), $p75^{NTR}$ does not possess catalytic activity but it recruits intracellular effectors depending on its *holo-* (NGF-bound) and *apo-* (unbound) forms (Roux and Barker, 2002). In those systems expressing both receptors, $p75^{NTR}$ can co-operate with trkA forming a "super affinity" receptor, thus enhancing the response to low concentrations of NGF (Huber and Chao, 1995).

In addition to expressing NGF, islets also express both trkA and $p75^{NTR}$, which are localised to β-cells (Vidaltamayo et al. 2003). Identification of NGF and its receptors within islets led to investigations on the existence of a possible autocrine loop, which indicated that NGF is released from rat islets in a glucose-dependent manner, up-regulates β-cell trkA expression (Rosenbaum et al., 1998), and plays a role in β-cell survival via trkA (Pierucci et al., 1991). It has been reported that individuals with type 2 diabetes possess elevated levels of circulating NGF (Kim et al., 2009) and streptozotocin-induced damage of β-cells in vivo and in vitro results in up-regulation of NGF secretion (Larrieta et al., 2006). These observations, together with the insulin secretagogue effects of NGF (Rosenbaum et al., 2001), suggest that increased local NGF levels following β-cell damage may be responsible for promoting cell survival and enhancing their insulin secretory capacity.

These earlier islet studies have mainly focused on the action of NGF signaling via trkA in static incubation experiments, and there is currently insufficient information available on the role of NGF in dynamic insulin secretion and on its signaling in islets via the less well studied $p75^{NTR}$. In the current study we have therefore used selective inhibitors of NGF action at both trkA and $p75^{NTR}$ in dynamic perifusion experiments with isolated mouse and human islets to provide new insights into the autocrine/paracrine loop signaling elicited by islet-derived NGF.

2. METHODS

2.1 Materials

Culture media, supplements and NGF (Sigma-Aldrich, UK; anti-NGF neutralising antibody (Alomone labs, Israel); Ro 08-2750 (Tocris, UK); TAT-PEP5 (Calbiochem, UK); RIPA buffer, PureProteome Protein G Magnetic Beads and Amicon Ultracel 3K centrifugal filters (Millipore, UK); protease and phosphatase inhibitor cocktail (Roche, UK); 5% fat-free milk solution (Applichem, UK); rabbit-anti-NGF, rabbit-anti-$p75^{NTR}$, rabbit-anti-RhoGDI antibodies, X-ray films (Santa Cruz Biotechnologies, USA); guinea pig-anti-insulin antibody and aqueous mounting media (Dako, UK); Alexa-fluor donkey-anti-rabbit 488-conjugated IgG, donkey-anti-guinea pig 594-conjugated IgG and 10% polyacrylamide gels (Life Sciences, UK); donkey-anti-rabbit HRP-conjugated IgG, ECL substrate, NGF E-max ELISA kit and RealTime-Glo™ Viability Assay (Promega, UK); BCA kit (Pierce, UK).

2.2 Mouse and human islets

Mouse islets were isolated from male ICR mice (20-25g) by collagenase digestion (Papadimitriou et al., 2007) and human islets were isolated from eleven non-diabetic donors (BMI: 29.9 ± 1.5; donor age: 49 ± 3 years; 5M, 6F) at the King's College Hospital Islet Transplantation Unit, with appropriate ethical approval (Huang et al., 2004). Islets were maintained at 37°C in RPMI supplemented with 10% fetal bovine serum, 2mM glutamine and 100U/ml/0.1mg/ml penicillin/streptomycin (mouse) or CMRL supplemented with 2% human albumin, 4mM glutamine, 2mM HEPES (pH 7.2-7.4), and 10mM nicotinamide (human).

2.3 MIN6 cells and pseudoislets

MIN6 β-cells were maintained (37°C, 5% CO_2) in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine and 100U/ml;0.1mg/ml penicillin/streptomycin. For some experiments pseudoislets were generated by culturing MIN6 cells for 7 days, essentially as described (Hauge-Evan et al., 1999), although uncharged Petri dishes were used rather than gelatin-coated ones.

2.4 Immunofluorescent detection of NGF

Non-pathological human pancreas samples were embedded into paraffin blocks and 5µm sections were boiled in 0.01M citric acid buffer (pH 6.0) for 2.5 minutes for antigen retrieval. Sections were blocked (0.1% donkey serum in PBS with 0.02% triton X-100, 1h, room temperature), incubated at 4°C overnight with rabbit anti-NGF (1:50) and guinea pig anti-insulin (1:200) then exposed to Alexa-fluor secondary antibodies (1:250) for 1h at room temperature. Nuclei were stained with DAPI and images were acquired using a Nikon eclipse TE2000-U microscope.

2.5 Static insulin secretion

Isolated islets were pre-incubated for two hours at 37°C in a saline buffer (Gey and Gey, 1936) containing 2mM glucose then groups of 3 islets (mouse), 5 islets (human) or 10 MIN6 pseudoislets were incubated in 250µL of medium supplemented with agents of interest for 1 hour, after which 200µL of supernatant was retrieved for assay of insulin content (Jones et al., 1988). When the anti-NGF neutralising antibody (AbNGF; 5µg/mL) or Ro 08-2750 (0.5µM or 10µM) were used, they were added during both the pre-incubation and incubation phases (Niederhauser et al., 2000; Caroleo et al., 2015).

2.6 Dynamic insulin secretion

Groups of 40 isolated mouse or 50 human islets were transferred to chambers containing 1µm pore-size nylon filters and perifused at a flow rate of 0.5ml/min at 37°C with a physiological salt solution (Gey and Gey, 1936) supplemented with agents of interest, essentially as described previously (Liu et al., 2009). When TAT-PeP5 was used, islets were pre-incubated for 20 minutes with 10µM before being transferred to the perifusion chambers. Perifusate samples were collected at 2-minute intervals and secreted insulin was measured by radioimmunoassay (Jones et al., 1988).

2.7 NGF secretion profiling

Groups of 200 isolated mouse or human islets were perifused, as described in section 2.6, initially with a salt solution supplemented with 2mM glucose, then with 20mM glucose for 20 minutes followed by a further 20 minutes with 20mM glucose in the presence of 500nM PMA, a PKC activator (Jones et al., 1989). Perifusates were collected every two

minutes on ice, and 10µL of each sample was used to profile insulin secretion. The remaining perifusates were concentrated by lyophilisation (mouse: 2-fold) or using Amicon 3K centrifugal filters (human: 10-fold) and NGF content was quantified with the NGF E-max ELISA kit, according to the manufacturer's instructions.

2.8 Real time monitoring of islet viability

The viability of mouse islets in culture following TAT-PeP5 treatment was evaluated using a realtime viability assay over 24h, according to the manufacturer's continuous monitoring protocol.

2.9 Immunoprecipitation of Rho-GDI

PureProteome Protein G magnetic beads were loaded with 2.5µg anti Rho-GDI primary antibody to immunoprecipitate Rho-GDI from 100µL of total MIN6 cell lysates. Proteins were fractionated by denaturing SDS polyacrylamide gel electrophoresis (10% gel, 45 minutes, 200V) (Carito et al. 2012), transferred onto 0.2µm pore nitrocellulose and probed overnight at room temperature with rabbit anti-$p75^{NTR}$ antibody (1:500) and rabbit anti-Rho-GDI antibody (1:500). The membrane was then incubated for 1h at room temperature with anti-rabbit HRP-conjugated secondary antibody (1:10,000) and exposed to X-ray film after addition of the ECL substrate.

2.10 Statistical analyses

Data are expressed as mean±SEM. The numbers of experiments and replicates are included in the figure legends. Statistical analyses were performed by Student's *t-tests* and differences were considered significant for p values <0.05.

3. RESULTS

3.1 NGF is secreted from islets but does not follow a biphasic profile

Dynamic perifusion of isolated mouse islets at 2mM glucose indicated that there is a basal release of NGF and insulin, with NGF being secreted at a rate of 3.5-5 fg $islet^{-1}$ min^{-1} and insulin at 1-2 pg $islet^{-1}$ min^{-1}. It can be seen from Figure 1a that during the first 10 minutes of exposure to 20mM glucose, there was a 6-8 fold rise in insulin output, which was accompanied by a 5-7 fold parallel increase in NGF secretion (t_{10}-t_{20}). Continued exposure to 20mM glucose (t_{20}-t_{30}) resulted in a sustained second phase of insulin secretion (approximately 4-5 fold basal), but NGF output declined towards basal levels. Exposure to the PKC activator PMA (t_{30}-t_{50}), triggered a rapid and sustained potentiation of glucose-stimulated insulin secretion, as expected (Jones et al., 1988), but it failed to produce a similar sustained increase in NGF secretion, despite two secretory peaks at t_{34} and t_{42}. Perifusion experiments were also performed using isolated human islets, but although insulin secretion in response to glucose and PMA was readily detectable (data not shown), the amount of NGF released remained below the detection limit of the ELISA kit for every batch of human islets assayed (n=6). This inability to detect NGF secretion was not a consequence of the absence of this peptide in human islets since immunofluorescence staining of human pancreas indicated NGF expression by islets, where it co-localised with insulin immunoreactivity (Figure 1b).

3.2 Disruption of NGF signaling stimulates basal insulin release in static incubations.

Identification of dynamic basal and glucose-stimulated NGF secretion from islets (Figure 1a) led us to initially perform static incubation studies to determine the contribution of NGF released from islets under non-stimulatory conditions (2mM glucose) to insulin secretion. In these experiments the biological activity of endogenously released NGF was neutralised with an anti-NGF antibody (AbNGF, 5µg/mL), while the ability of endogenous NGF to bind to its

receptors was impaired by supplementing the incubation medium with Ro 08-2750 (Ro) at 0.5 and 10μM to disrupt binding to $p75^{NTR}$ and both trkA and $p75^{NTR}$, respectively (Niederhauser et al., 2000). As shown in Figure 2a, mouse islets responded to NGF neutralisation by significantly increasing insulin secretion at 2mM glucose, and similar effects were observed following disruption of NGF binding to its receptors. Although we could not detect NGF secretion from human islets in perifusion experiments, a role for endogenous NGF in regulating insulin secretion was evident from the capacity of AbNGF and Ro to stimulate insulin secretion at 2mM glucose (Figure 2b), in a similar manner to that seen in mouse islets (Figure 2a). We confirmed that the increased insulin secretion observed with AbNGF was a direct consequence of changes in the secretory response of β-cells rather than via paracrine signaling of endogenous NGF through α- and/or δ-cells since MIN6 pseudoislets, which are composed entirely of β-cells, also responded with elevated insulin secretion when incubated with AbNGF at 2mM glucose (Figure 2c). The effects observed using AbNGF were dependent on neutralisation of endogenous NGF as exposure to 5μg/mL isotypic IgG rather than AbNGF did not induce any changes in insulin secretion from either mouse or human islets (Figure 2d).

3.3 Blockade of NGF signaling stimulates dynamic basal insulin release and reduces glucose-stimulated insulin secretion.

The data shown in Figures 2a and 2b are indicative of a negative feedback effect of locally released NGF on basal insulin secretion, which is not further modified by the addition of 50ng/mL exogenous NGF. The potential role of NGF in regulating the capacity of islets to show appropriate insulin secretion in response to a maximal stimulatory concentration of glucose (20mM), and further investigation of dynamic effects at 2mM glucose, were investigated using the multichannel perifusion system that we had used for quantification of NGF release since this represents an ideal approach for evaluation of the onset, amplitude and reversibility of insulin secretory responses. Perifusion of mouse islets with 0.5μM Ro, to block interaction of endogenous NGF with $p75^{NTR}$ (Figure 3a), or 10μM Ro, to inhibit endogenous NGF binding to both trkA and $p75^{NTR}$ (Figure 3b) resulted in significant elevation of basal insulin secretion, confirming the data obtained in the static incubation experiments (Figure 1a). Exposure of islets to 0.5μM Ro in the presence of 20mM glucose resulted in an insulin secretory profile virtually superimposable with that seen when islets were perifused with medium containing 20mM glucose alone (Figure 3a). However, it can be seen from Figure 3b that inhibition of $p75^{NTR}$ and trkA with 10μM Ro caused a significantly delayed onset of glucose-stimulated insulin secretion, with a peak response 20 minutes after the increase in glucose concentration, rather than the immediate increase seen in the presence of 20mM glucose alone. In contrast, perifusion of islets at 20mM glucose in the presence of exogenous NGF (50ng/mL) did not affect the rate of onset of insulin secretion, but islets showed a significantly higher amplitude of insulin release than those perifused in the absence of NGF (Figure 3c). This was most apparent during the last 10 minutes of exposure to NGF (t_{40}-t_{50}), a time course over which the concentration of endogenously secreted NGF physiologically declined, according to the profile shown in Figure 1a.

The increase in basal insulin release upon inhibition of endogenous NGF action was confirmed in separate protocols where islets were perifused in the presence of 0.5μM or 10μM Ro (Figure 4a), and the elevation in secretion at 2mM glucose was transient and rapidly reversible upon withdrawal of the inhibitor. A role for basal NGF secretion in determining the rate of onset and amplitude of the insulin secretory response to 20mM glucose also emerges from the data in Figure 4b: when mouse islets were pre-exposed to 10μM Ro in the presence of 2mM glucose for the last 10 minutes of the pre-perifusion, the subsequent glucose-stimulated insulin secretion was impaired such that there was a delay in the first phase, even though the inhibitor was not present during the glucose challenge.

3.4 Disruption of $p75^{NTR}$ activity impairs glucose-stimulated insulin secretion.

Given that $p75^{NTR}$ is able to signal differently depending to whether it is bound or not to NGF (in its *holo*- or *apo*-forms) (Roux and Barker, 2002), further perifusion experiments were performed using the $p75^{NTR}$-selective inhibitor TAT-PeP5, which binds covalently to the intracellular domain (ICD) of the receptor (Yamashita and Tohyama, 2003), thus blocking its activity regardless of the presence of NGF. Treatment with TAT-PeP5 for 20 minutes prior to perifusion induced significant reductions in glucose-dependent insulin secretion from mouse (Figure 5a) and human (Figure 5b) islets. This inhibition was not due to TAT-PeP5 exerting deleterious effects on islet survival since continuous monitoring of islet viability for 24 hours after TAT-PeP5 exposure indicated that there was no difference compared to the profile obtained with control islets that had not been incubated with TAT-PeP5 (Figure 5c). In these experiments the reductions in glucose-stimulated insulin secretion in response to TAT-PeP5 treatment were similar in mouse and human islets, but there were differential effects on basal insulin secretion at 2mM glucose. Thus, as shown in Figure 5a and 5b, basal insulin release from TAT-PeP5-treated mouse islets was significantly higher than control, but it was not significantly different in human islets ($p>0.2$, AUC measurements of TAT-PeP5-treated vs control), perhaps a consequence of the higher basal secretion rate observed in the human islets.

3.5 $p75^{NTR}$ interacts with Rho-GDI in β-cells.

$p75^{NTR}$ does not directly regulate intracellular enzyme activity, but it interacts with intracellular proteins such as Rho-GDI, an inhibitor of Rho family GTPases, to influence cytosolic dynamics in a tissue-specific manner (Caroleo et al, 2015; Yamashita and Tohyama, 2003; Sachs et al, 2007; Deponti et al., 2009; Bilderback et al, 1999; Nevins and Turmon, 2006; Kowluru and Veluthakal, 2005). Rho-GDI has been implicated in insulin granule movement (Rorsman and Renström, 2003), and immunoprecipitation of this 21kDa protein in MIN6 β-cells followed by western blotting indicated that the low affinity 75kDa NGF receptor can interact directly with Rho-GDI (Figure 6).

4. DISCUSSION

It has been known for some time that rodent islet β-cells secrete NGF upon appropriate secretagogue stimulation (Rosebaum et al., 1998), but earlier experiments did not provide information on the time-course of NGF secretion, nor whether its release coincided with that of insulin. The dynamic perifusion studies presented here have demonstrated concomitant NGF and insulin secretion from mouse islets during the first 10 minutes of a glucose challenge, suggesting that release of NGF-containing granules occurs together with readily releasable insulin secretory granules during the first phase of secretion (Rorsman and Renström, 2003). Continued exposure to 20mM glucose induces insulin granule movement towards the plasma membrane, but under these circumstances of the slower, more sustained insulin secretory output NGF release from perifused mouse islets declined. We used the phorbol ester PMA to promote robust potentiation of glucose-stimulated insulin secretion and this provided further evidence of uncoupling of insulin and NGF secretion, similar to a previous observation in static incubation of rat islet cells where stimulation with the insulin secretagogue dbcAMP failed to increase NGF (Rosenbaum et al., 1998).

In contrast to our observations with mouse islets, we were unable to profile dynamic NGF secretion from human islets, as the amount of NGF released under all conditions was below the immunoassay detection limit of 7.8pg/mL, even when perifusates were concentrated 10-fold. However, immunohistochemical staining of human pancreas indicated that NGF was present in the insulin-containing cells and the increase in insulin secretion from human islets in response to a neutralising NGF antibody are consistent with NGF being secreted from human islets to have autocrine effects. Thus, it

is likely that NGF is released at lower levels in human islets than in mouse islets such that it does not reach sufficient concentrations in the perifusate, even when concentrated, for reliable detection by ELISA.

NGF release from mouse islets accompanied basal insulin secretion at 2mM glucose and also during the first phase of glucose-stimulated insulin secretion, and neutralisation of endogenous NGF elevated basal insulin secretion from both mouse and human islets. These observations are consistent with islet-derived NGF contributing to the basal insulin secretion tone, but our data indicating that inhibition of endogenous NGF signaling reduced glucose-stimulated insulin secretion suggests that the NGF autocrine loop operates differentially at basal glucose and after islets are stimulated by elevations in glucose concentration. This difference might be reflected in the roles of the two receptors in mediating the effects of NGF, and the use of Ro to selectively inhibit NGF interaction with the high (trkA) and low ($p75^{NTR}$) affinity receptors allowed us to investigate the contribution of these receptors to basal and glucose-stimulated insulin secretion.

Dynamic assessment of insulin secretion using Ro confirmed the inhibitory role played by endogenous NGF on insulin secretion at 2mM glucose, and these experiments also demonstrated impairment of glucose-stimulated insulin secretion upon perturbation of NGF signaling. In particular, the use of 10μM Ro to block NGF binding to both receptors (Niederhauser et al., 2000) delayed the insulin secretory response to glucose such that the rapid first phase was lost. In contrast, exogenous NGF potentiated glucose-stimulated insulin secretion, suggesting that the transient elevation of endogenous NGF may have a physiological role to prevent hyperglycaemia. Indeed, it has been reported that an increase in circulating NGF occurs during type 2 diabetes (Kim et al., 2009), which may be a compensatory mechanism to maintain normoglycaemia. These results are also in agreement with an earlier report that acute and 5 days stimulation with NGF increased insulin secretion from rat β-cells (Rosenbaum et al., 2001). However, continuous stimulation of β-cells with high circulating levels of NGF coupled to a higher than normal glucose concentration could be a pathological mechanism driving the loss of β-cell secretory capability in the longer term.

Thus, our data are consistent with NGF not only contributing to the maintenance of low levels of insulin release in the absence of a glucose stimulus, but also being involved in the fine tuning of the glucose-dependent insulin secretory response. In addition, basal NGF secretion from islets also contributes to the amplitude of first phase insulin secretion *per se* since interfering with its binding to trkA and/or $p75^{NTR}$ through pre-exposure of islets to Ro reduced their glucose-stimulated insulin output. These direct, rapid effects of signaling via NGF receptors in islets to promote glucose-induced insulin secretion are complementary to the long-term effects of NGF to up-regulate mRNA encoding voltage-gated sodium channels in rat β-cells and increase β-cell electrical activity (Vidaltamayo et al., 2002), and its ability to increase current through L-type Ca^{2+} channels in β-cells (Rosenbaum et al., 2002).

The majority of NGF-related islet studies published so far have focused on the long-term effects of NGF signaling via trkA, and they were aimed at identifying its role in β-cell survival and proliferation (Rosenbaum et al., 1998; Vidaltamayo et al., 2003; Pierucci et al., 1991; Hata et al., 2015). The low affinity receptor, $p75^{NTR}$, has been implicated in regulating neuronal apoptosis and regeneration (Taborsky et al., 2014), control of glucose homeostasis via effects on skeletal muscle (Baeza-Raja et al., 2005) and in mediating islet responses to the GLP-1 analogue exendin-4 (Gezginci-Oktayoglu and Bolkent, 2009), and regulating cAMP levels independently of NGF (Baeza-Raja et al., 2016). In terms of receptor actions, $p75^{NTR}$ recruits effectors via its intracellular C-terminal domain (ICD) and a role for its correct cycling between the *apo*- and *holo*- forms in islets, and the consequent switch of intracellular partners, emerges from our dynamic profiling data in which TAT-PeP5 was used to inhibit $p75^{NTR}$. This peptide blocks the ability of $p75^{NTR}$ to interact with intracellular partners, impairing the effect of its cycling between the *apo*- and *holo*- forms, thus releasing into the cytosol its ICD partners and preventing the ICD-mediated recruitment of other effectors (Baeza-Raja et al., 2005), irrespective of the availability of NGF in the extracellular environment. TAT-PeP5 treatment resulted in the loss of 50-60% of the insulin

secretory response of both mouse and human islets to glucose, providing evidence for a role of $p75^{NTR}$ in the full amplitude of glucose-stimulated insulin secretion.

Effectors that are recruited by $p75^{NTR}$, including caveolin, small GTPases and their GDP dissociation inhibitors (GDI), have been implicated in β-cell secretory granule exocytosis (Bilderback et al., 1999; Muragaki et al., 1995; Wang and Thurmond, 2007). In particular, caveolin was identified as a CDC42-GDI in β-cells (Kowluru and Veluthakal, 2005), and $p75^{NTR}$ is reported to bind both caveolin and Rho-GDI in PC12 pheochromocytoma cells through portions of its ICD domain (Yamashita and Tohyama, 2003; Bilderback et al., 1999). Our immunoprecipitation studies have now demonstrated physical interaction between $p75^{NTR}$ and Rho-GDI in mouse β-cells, providing a basis for insulin granule movement in β-cells being downstream of $p75^{NTR}$ bound to Rho-GDI. Our TAT-PeP5 experiments support this model, where blockade of the $p75^{NTR}$ ICD would be expected to reduce glucose-stimulated granule translocation because of the impaired interaction between the receptor and Rho-GDI, and the data are consistent with $p75^{NTR}$ cycling being important in delivering the correct amplitude of insulin secretion in response to a glucose challenge.

5. **Conclusions.**

The role of the autocrine NGF loop in islet β-cells goes beyond the canonical survival pathways promoted by this peptide. The NGF secretion profiling data, where there is a basal, tonic release and transient elevation following a glucose challenge, together with the insulin secretion data revealing that endogenous NGF inhibits basal insulin release while promoting glucose-stimulated insulin secretion suggest an autocrine role for NGF in the regulation of insulin secretion. We propose that NGF release allows fine-tuning of the β-cell secretory activity to regulate both basal insulin secretion and the appropriate biphasic profile of glucose-stimulated insulin secretion. The role played by $p75^{NTR}$ at stimulatory concentrations of glucose is of particular interest as impairment of its cycling between the *apo* (NGF un-bound) and *holo* (NGF bound) forms impacts severely on the secretory response to glucose, most likely through the inability of $p75^{NTR}$ to recruit intracellular mediators controlling β-cell secretory granule movement.

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Contribution statement

Experiments were designed by AP, MCC and SP, material was collected by RCC and GH and experiments were conducted by AP. Data were analysed by AP and SP and interpreted by AP, SP, GH, RCC, MCC and EC. AP and SP drafted the manuscript, and all authors have agreed to the final version of the manuscript.

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Figure Legends:

Figure 1: NGF and insulin secretion and expression in islets

a) Dynamic insulin and NGF secretion from 200 mouse islets was quantified using specific immunoassays. Results are expressed as fold increase over basal peptide release of 6 independent experiments (mean±SEM). b) Human pancreas sections were immunoprobed with antibodies directed against insulin (red) and NGF (green) and nuclei were stained with DAPI (blue). Islet cells in which insulin and NGF are co-localised are indicated by yellow fluorescence in the merged image. The image is representative of 3 independent human pancreas sections.

Figure 2: Effect of inhibition of endogenous NGF signaling on basal insulin secretion

Group of 3 mouse islets (a, d), 5 human islets (b, d) or 10 MIN6 pseudoislets (c) were incubated for one hour in the presence of the agents shown and insulin release was quantified by radioimmunoassay. Results are expressed as mean+SEM of 6 independent experiments in a) and c), and 3 independent experiments in b) and d), with 6-8 replicates per group. *p<0.05, **p<0.01, ***p<0.001 versus 2mM glucose. AbNGF: neutralising NGF antibody; Ro: Ro 08-2750; IgG: isotypic IgG.

Figure 3: Effect of blocking NGF binding to trkA and/or $p75^{NTR}$ on insulin secretion

Groups of 40 mouse islets were perifused with buffers supplemented with 0.5µM Ro (a), 10µM Ro (b) or 50ng/mL NGF (c) and insulin secretion was quantified by radioimmunoassay. Area under the curve (AUC) data: t_0-t_{70} full profile; t_0-t_{30} 2mM glucose (basal); t_{32}-t_{50} glucose-stimulated insulin secretion divided into first phase (t_{32}-t_{40}) and second phase (t_{42}-t_{50}); t_{52}-t_{70} 2mM glucose (final). Data are expressed as mean±SEM of 4 separate groups of islets, representative of 4 independent experiments. In the perifusion profiles *p<0.05 and #p<0.01 vs the same time points of the controls, and in the AUC data *p<0.05 *vs* the same section of the control.

Figure 4: Impairment of NGF binding to trkA and/or $p75^{NTR}$ reduces glucose-stimulated insulin secretion

Groups of 40 mouse islets were perifused with buffers supplemented with 2mM glucose in the absence (solid lines) or presence (dotted lines) of Ro. a) perifusion of mouse islets in the presence of 0.5μM and 10μM Ro at 2mM glucose. b) mouse islets were pre-exposed to 10μM Ro during the last 10 minutes of the pre-perifusion hour (dotted line) then challenged with 20mM glucose. Area under the curve (AUC) data: a) t_{10}-t_{24} 0.5μM Ro; t_{26}-t_{40} 10μM Ro; b) t_0-t_{40} full profile; t_{12}-t_{40} glucose-stimulated insulin secretion divided into first phase (t_{12}-t_{28}) and second phase (t_{30}-t_{40}). Data are expressed as mean±SEM of 6 separate groups of islets, representative of 2 independent experiments. In the perifusion profiles *p< 0.05, # p<0.01 *vs* the same time points of the controls and in the AUC data *p<0.05 and ** p<0.01 *vs* the same section of the control.

Figure 5: Effect of $p75^{NTR}$ intracellular blockade on glucose-stimulated insulin secretion

Groups of 40 mouse islets (a) or 50 human islets (b) were pre-treated for 20 minutes in the absence or presence of 10μM TAT-PeP5 then perifused with buffers supplemented with the agents shown. Area under the curve (AUC) data: t_0-t_{30} full profile; t_0-t_{10} 2mM glucose (basal); t_{12}-t_{30} glucose-stimulated insulin secretion; c) real time viability measurements of mouse islets (mean±SEM, n=10) in culture for 24h in the absence or presence of 10μM TAT-PeP5. Perifusion results are expressed as mean±SEM of 3-4 separate groups of islets, representative of 3 (human) or 4 (mouse) independent experiments. In the secretory profiles (a, b) and in c *p<0.05 and # p<0.01 *vs* the corresponding control data. In the AUC graphs, *p<0.05 and **p<0.01 *vs* the same section of the control.

Figure 6: $p75^{NTR}$ interacts with Rho-GDI in MIN6 β-cells

The image shows immunoblotting for $p75^{NTR}$ (upper panel, 75kDa) and Rho-GDI (lower panel, 21kDa) in protein extracts from MIN6 cell total lysate (TL) and immunoprecipitated (IP) Rho-GDI, and indicates that Rho-GDI binds to $p75^{NTR}$.